Virology 443 (2013) 226–235

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Failure in activation of the canonical NF-κB pathway by human T-cell leukemia virus type 1 Tax in non-hematopoietic cell lines

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ARTICLE INFO

Article history:
Received 21 January 2013
Returned to author for revisions
25 February 2013
Accepted 29 April 2013
Available online 18 June 2013

Keywords:
HTLV-1
Tax1
NF-κB
Canonical pathway
Transcription
Hematopoietic cells

ABSTRACT

Human T-cell leukemia virus type 1 (HTLV-1) Tax (Tax1) plays crucial roles in leukemogenesis in part through activation of NF-κB. In this study, we demonstrated that Tax1 activated an NF-κB binding (gpκB) site of the gp34/OX40 ligand gene in a cell type-dependent manner. Our examination showed that the gpκB site and authentic NF-κB (IgκB) site were activated by Tax1 in hematopoietic cell lines. Non-hematopoietic cell lines including hepatoma and fibroblast cell lines were not permissive to Tax1-mediated activation of the gpκB site, while the IgκB site was activated in those cells in association with binding of RelB. However RelA binding was not observed in the gpκB and IgκB sites. Our results suggest that HTLV-1 Tax1 fails to activate the canonical pathway of NF-κB in non-hematopoietic cell lines. Cell type-dependent activation of NF-κB by Tax1 could be associated with pathogenesis by HTLV-1 infection.

Introduction

Infection with human T-cell leukemia virus type1 (HTLV-1) causes adult T-cell leukemia (ATL) and inflammatory disorders such as HTLV-1 associated myelopathy/tropical spastic paraparesis and HTLV-1 associated uveitis (Hinuma et al., 1981; Osame et al., 1986; Poiesz et al., 1980). HTLV-1 encodes Tax1, which has been shown to be implicated in the pathogenesis of HTLV-1 associated disorders (Giam and Jeang, 2007). Tax1 is a trans-acting transcriptional regulator that exerts its function via cellular transcription factors mainly cAMP responsive element binding protein (CREB), nuclear factor κB (NF-κB) and serum responsive factor (SRF) (Ballard et al., 1988; Fujii et al., 1992; Lenzmeier et al., 1998). Tax1 interaction with cellular transcription factors leads to transcriptional changes in many cellular genes as well as activation of HTLV-1 transcription (Lenzmeier et al., 1998; Ohtani and Nakamura, 2002; Yoshida M., 2001; Zhao and Giam, 1992). Previous studies demonstrated that activation of NF-κB by Tax1 is closely associated with development and maintenance of ATL (Akagi et al., 1995; Ben-Neriah and Karin, 2011; Matsuoka and Jeang, 2007; Qu and Xiao, 2011). Tax1 activates both the NF-κB canonical and non-canonical pathways consisting of RelA with p50 and RelB with p52, respectively (Harhaj and Harhaj, 2005; Xiao et al., 2001). Activation of the canonical pathway results mainly from association of Tax1 with the IKK complex, leading to inactivation of IκB by phosphorylation, followed by translocation of the RelA/p50 complex to the nucleus (Geleziunas et al., 1998; Jin et al., 1999). Tax1 is also able to directly facilitate the transition of the precursor p100 into p52, thus promoting translocation of the non-canonical pathway complex RelB with p52 to the nucleus (Higuchi et al., 2007; Shoji et al., 2009). In the nucleus, NF-κB binds promoter DNA elements to initiate or enhance transcription of respective genes. The other member of the HTLV family is HTLV-2, which also produces a trans-acting transcriptional molecule so-called Tax2 (Ross et al., 1996). Tax2 does resemble Tax1 in terms of the ability to activate NF-κB and differs from Tax1 in that Tax2 predominantly activates the canonical NF-κB pathway (Higuchi et al., 2007; Shoji et al., 2009).

The gp34 gene was first identified to be a target of Tax1 in human T cells (Miura et al., 1991; Tanaka et al., 1985). Since its discovery, the gene product has been revealed as an OX40 ligand (OX40L), a type II transmembrane member of the tumor necrosis factor (TNF) superfamily, which is expressed on antigen presenting

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http://dx.doi.org/10.1016/j.virol.2013.04.032

cells, endothelial cells and activated T cells under normal immune conditions (Baum et al., 1994). OX40L interacts with OX40, a member of the TNF receptor family, which is predominantly expressed in T cell and delivers co-stimulatory signals implicated in expansion, survival and homeostasis of T cells (Ishii et al., 2010). Among ATL cells and HTLV-1 infected T cells, only T cells expressing Tax1 express OX40L on their cell surface. We previously reported that the gp34 (OX40L) gene promoter has an element capable of NF-κB binding (Ohtani et al., 1998). The NF-κB binding (gpκB) site is, at least in part, responsible for Tax1-mediated expression of OX40L on T cells. This Tax1-mediated expression makes the interaction of OX40 with OX40L possible in the same T cells. OX40 and OX40L interaction in ATL development remains to be examined. Our preliminary results indicate that the gpκB site in the OX40L promoter is somewhat different from the classical NF-κB binding site represented by the NF-κB binding site (IgκB site) of the immunoglobulin light chain gene promoter. The gpκB site in the OX40L promoter fails to be activated in the presence of Tax1 in the fibroblast cell line REF52, in contrast to activation in the T cell line Jurkat.

In this study, we wished to understand how Tax1 activated the gpκB site in a cell type-dependent manner. Our results indicate that Tax1 does not activate the canonical NF-κB pathway in non-hematopoietic cell lines, suggesting involvement of Tax1 in T cell pathogenesis.

Results

Tax1 does not activate the gpκB site in non-hematopoietic cells

Tax1 activated the IgκB and gpκB sites in Jurkat cells (Fig. 1), as shown previously (Ohtani et al., 1998). Unexpectedly, in REF52 cells, Tax1 did not activate the gpκB site, while the IgκB site was activated (Fig. 1). We hypothesized that responses of the gpκB site to Tax1 might be cell lineage-dependent. To test this notion, more cell lines of hematopoietic and non-hematopoietic lineages were used for reporter assays with Tax1. Non-hematopoietic cell lines, the human osteosarcoma cell line MG63, the human hepatoma cell lines Huh7 and HepG2, the human cervical cancer cell line HeLa, the human embryonic kidney cell line 293T and the murine neuroblastoma cell line C-1300 exhibited Tax1-mediated activation of the IgκB site, but the gpκB site was not activated in response to Tax1 in those cell lines (Fig. 1). A slight but not significant activation of the gpκB site by Tax1 was observed in HeLa and 293T cells, however this activation may be due to indirect effects of Tax1 (discussed later). Hematopoietic cell lines, the human Burkitt's lymphoma cell line Raji and the human myelogenous leukemia cell line K562, allowed activation of the gpκB site by Tax1 similar to Jurkat (Fig. 1). These results suggest that Tax1 does not activate the gpκB site in non-hematopoietic lineage cell lines.

In order to study cell type-dependent activation of the gpκB site by Tax1, we examined effects of ectopic expression of NF-κB subunits (RelA, p105/p50, RelB, p100/p52 and c-Rel) on the activation of the gpκB site in non-hematopoietic cell lines. The gpκB site was activated by overexpression of either RelA or p50 without Tax1 in REF52, MG63 and HeLa cells (Fig. 2A). Similarly RelA-dependent activation was seen with the IgκB site (Fig. 2A). Increased activation by RelA was dose-dependent (Fig. S1). Ectopic expression of c-Rel slightly activated the gpκB site (Fig. 2A). Introduction of RelB or p52 combination however did not change reporter gene expression from the gpκB site, while the IgκB site was activated by treatment with RelB (Fig. 2A and B). These results suggest that the canonical pathway is closely associated with activation of the gpκB sites in non-hematopoietic cells.

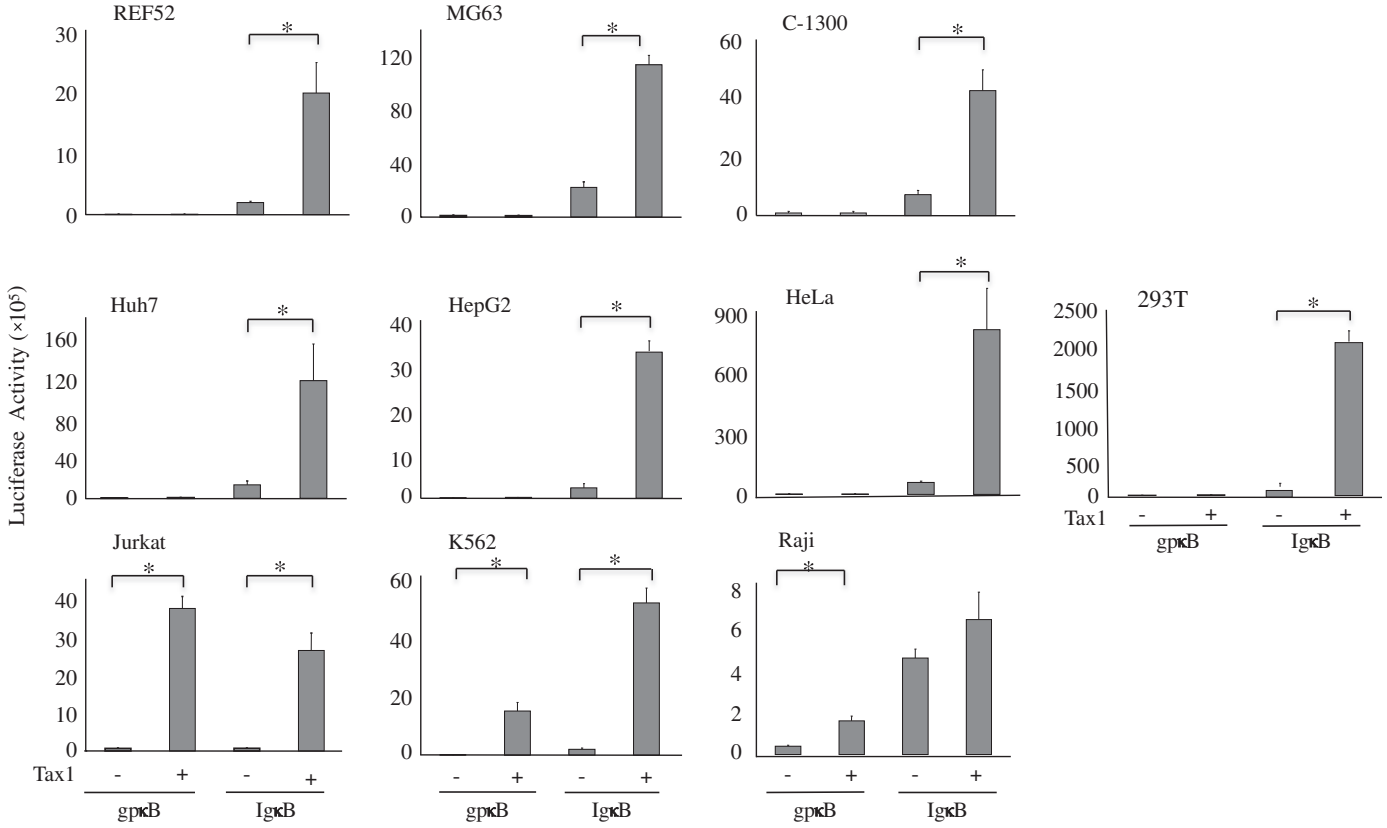

Fig. 1. Cell type-dependent activation of the gpκB site by Tax1. Cells were transfected with the gpκB or IgκB reporter plasmid along with pMT-2Tax. REF52, MG63, HeLa, 293T, Jurkat, K562 and Raji cells were cultured for 48 h. Huh7, HepG2 and C-1300 cells were cultured for 24 h and harvested for luciferase activity determination. Luciferase activity was normalized to protein content. Data are means ± SE. $^{*}P < 0.05$.

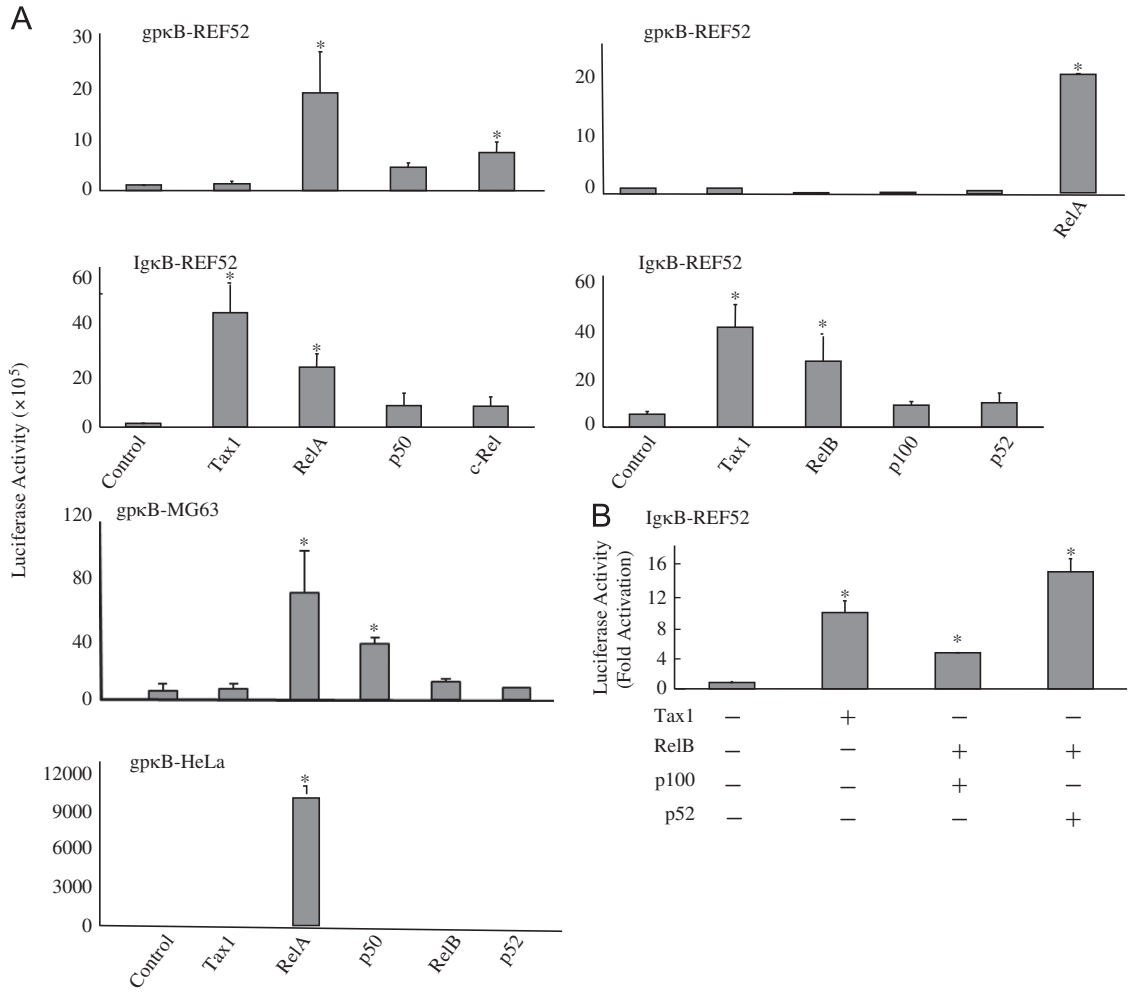

Fig. 2. Effects of NF-κB subunits on gpκB site activation. Expression plasmids for NF-κB subunits or Tax1 were transfected to REF52, HeLa and 293T cells along with the gpκB or IgκB reporter plasmid. Cells were cultured for 48 h and harvested for luciferase activity determination. Luciferase activity was normalized to protein content (A) or to β-galactosidase activity (B). Data are means ± SE. $^{*}P < 0.05$.

Tax1 activates the non-canonical NF-κB pathway in REF52 cells

Based on these results, we assumed the possibility that Tax1 did not activate the canonical pathway in non-hematopoietic cell lines and that the non-canonical pathway might be responsible for Tax1-dependent activation of the IgκB site. The assumption was examined by experiments with REF52 cells overexpressing the NF-κB subunits. Interestingly, overexpression of the non-canonical pathway subunit RelB alone or along with p52, a partner in the non-canonical pathway, significantly activated the IgκB sites in REF52 cells in the absence of Tax1 (Fig. 2A and B). Consistent with this, disruption of RelB or p52 by introduction of respective siRNA significantly reduced activation of the IgκB site by Tax1, while siRNA for RelA or p50 did not show appreciable effect (Fig. 3).

HTLV-2, a human retrovirus close to HTLV-1, produces Tax2, which is known to predominantly activate the canonical NF-κB pathway (Matsumoto et al., 1997). Similar to Tax1, Tax2 activated both gpκB and IgκB sites in Jurkat cells, while, in REF52 cells, the gpκB site was not activated by Tax2 (Fig. 4A). Tax2 exhibited significantly lower activation of the IgκB sites than Tax1 in REF52 cells (Fig. 4B). A chimera Tax mutant (Tax1/2) between Tax1 and Tax2, in which the Tax1 (225–232) region responsible for activation of the non-canonical pathway was replaced with a region of Tax2 (225–232), has little ability to activate the non-canonical pathway (Shoji et al., 2009). The effects of the Tax1/2 mutant on activation of the gpκB and IgκB sites were close to those of Tax2, rather than Tax1, in Jurkat and REF52 cells (Fig. 4). These results strongly indicate the possibilities that the gpκB site is activated by the canonical, but not non-canonical, pathway and that the canonical pathway is scarcely activated by Tax1 in non-hematopoietic cell lines.

RelA and p50 are expressed in non-hematoietic cells

We therefore examined expression and localization of NF-κB subunits in non-hematopoietic cells. Immunostaining and Western blot analyses clearly showed that REF52, MG63 and HeLa cells expressed RelA, p50, RelB and p52 (Fig. 5A–H). RelA was mainly present in the cytoplasm, while p50 was in the nucleus and cytoplasm (Fig. 5A–D). The ability of those subunits to bind κB sites was examined by electrophoreitic mobility shift assay (EMSA). In contrast to the cytoplasmic extracts which gave supershift bands with anti-RelA antibody, nuclear extracts from REF52 cells with or without Tax1 expression did not form complexes containing RelA with the gpκB site and the IgκB site (Fig. 6A and B). Binding of p50 was seen with both nuclear and cytoplasmic extracts; nuclear extracts from Tax1-expressing REF52 cells formed the most abundant complex. Ectopic expression of RelA in REF52 cells induced its complex formation with the gpκB site (Fig. 6C). Complexes containing RelA were also detected using nuclear extracts from REF52 cells ectopically expressing p50. These results are consistent with reporter assays. RelB, p52 and c-Rel were not included in complexes

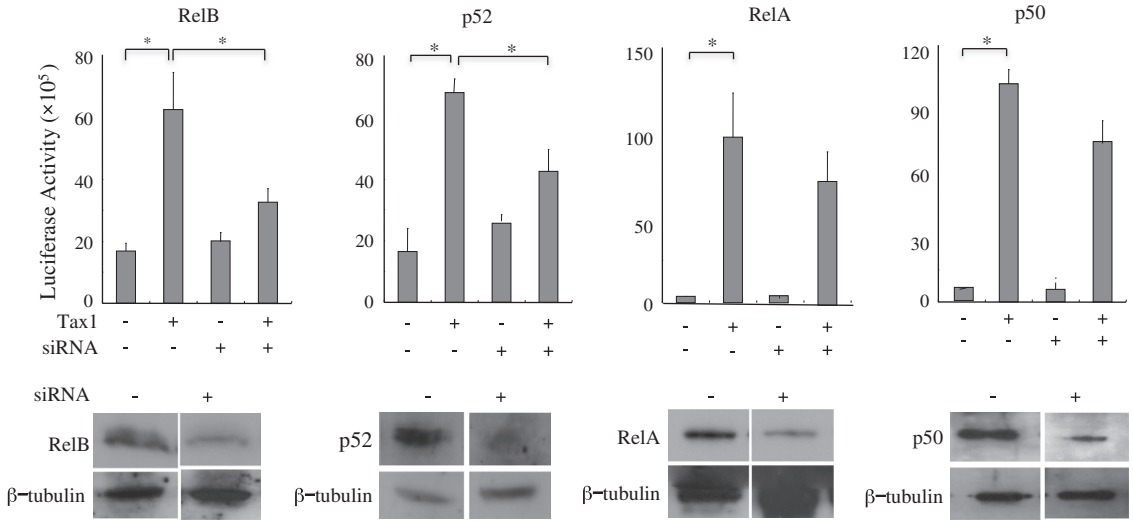

Fig. 3. Effects of siRNA for RelB, p52, RelA and p50 on κB site activation. REF52 cells were transfected with siRNA (10 nM) for RelB, p52, RelA and p50, and, one day after, introduced with pIgκB-Luc with or without pMT-2Tax in Lipofectamine 2000. Cells were harvested for luciferase activity determination 24 h after transfection with the reporter plasmid. Luciferase activity was normalized to protein content. Data are means ± SE. $^{*}P < 0.05$. Western blotting with antibodies to RelB, p52, RelA, p50 and β-tubulin was performed with lysates from cells transfected with siRNA.

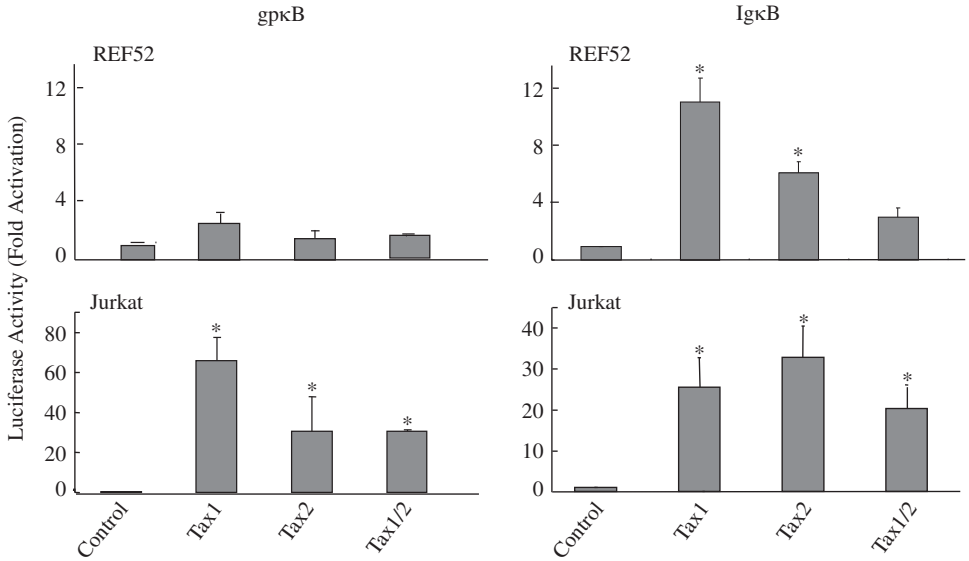

Fig. 4. Effects of Tax2 on gpκB site activation. REF52 and Jurkat cells were transfected with Tax1, Tax2 or Tax1/2 expression plasmid along with the gpκB or IgκB reporter plasmid and pCMV-β-gal. Cells were cultured for 48 h and harvested for luciferase activity determination. Luciferase activity was normalized to β-galactosidase activity. Data are means ± SE. $^{*}P < 0.05$.

formed with the gpκB site when nuclear extracts were prepared from Tax1-expressing REF52 cells (Fig. S2).

lmmunofluorescence staining confirmed the results of EMSA. Tax1 introduction did not appreciably alter localization of RelA predominantly localized in the cytoplasm in those cells except for HeLa cells (Fig. 5A, C and D). In HeLa cells after Tax1 transfection, RelA was seen in the nucleus as well as the cytoplasm irrespective of Tax1 expression. This RelA expression might be attributable to Tax1-dependent paracrine mechanism, because HeLa cells even without Tax1 expression expressed RelA in the nucleus as indicated by arrowheads in Fig. 5D. The p50 molecule was mainly seen in the peri-nuclear region. RelB was present in the cytoplasm before introduction of Tax1 like RelA. In contrast to RelA, Tax1 facilitated the influx of RelB to the nucleus. Immunostaining with anti-p52 antibody detected p100/p52 mainly in the cytoplasm without Tax1 and in both the cytoplasm and nucleus with Tax1. Western blot examination also showed that RelB and p52 were seen in the nucleus after Tax1 was expressed, while RelA stayed in the cytoplasm even after Tax1 introduction in REF52 and MG63 cells (Fig. 5E and G). HeLa cells showed RelA translocation to the nucleus, as discussed before (Fig. 5H). In contrast, Jurkat cells exhibited translocation of RelA, as well as RelB and p52, in a Tax1-dependent manner (Fig. 5B and F).

RelA and p50 expressed in REF52 cells were examined for their function by stimulation with tumor necrosis factor α (TNFα). TNFα induced activation of the IgκB site in the luciferase reporter assays in association with translocation of RelA from the cytoplasm to the nucleus and phosphorylation of IκBα potentially leading to degradation (Fig. 5I–K). MG63 and HeLa cells also showed TNFα-induced RelA translocation (Fig. 5G and H). These

results suggest that RelA/p50 is functional in those non-hematopoietic cell lines.

Tax1 induces RelB and p52 binding to the IgκB site in vivo

In order to examine the ability of the IgκB site to bind NF-κB subunits in cells, we performed the chromatin immunoprecipitation (ChIP) assays in REF52, MG63 and HeLa cells with or without Tax1 expression (Fig. 7A, C and D). After reporter plasmid transfection with or without the Tax1 expression plasmid, cells were lysed and immunoprecipitated by anti-NF-κB subunit antibodies. None of the antibodies used precipitated appreciable gpκB and IgκB site DNA elements without Tax1 expression (Fig. 7A). Immunoprecipitation with anti-RelB, anti-p52 and anti-p50 antibodies detected the IgκB site with Tax1 expression. In addition, HeLa cells showed RelA binding to the IgκB site (Fig. 7D). As expected, only anti-p50 antibody precipitated the gpκB site in REF52 cells with Tax1 expression. Antibodies to RelA, RelB and p52 did not effectively precipitate the gpκB site in REF52 cells. These results indicate that at least REF52 and MG63 cells do not have the active form of the RelA/p50 complex in the nucleus in the presence of Tax1. In Jurkat cells, both the gpκB and IgκB sites were immunoprecipitated with all antibodies used in a Tax1-dependent manner (Fig. 7B).

Discussion

The present study demonstrates that the gpκB site is activated by HTLV-1 Tax1 in a cell type-dependent manner. Hematopoietic cell lines Jurkat, Raji and K562 activated the gpκB site upon Tax1 expression, whereas the seven non-hematopoietic cell lines failed to activate the gpκB site. One the other hand, the IgκB site was activated by Tax1 in both hematopoietic and non-hematopoietic cell lines used in this study. As overexpression of the canonical pathway subunit RelA activated the gpκB in non-hematopoietic cells, the gpκB unresponsiveness is presumably due to inactivation of the NF-κB canonical pathway by Tax1 in those cell lines. These results also suggest that the gpκB site is probably activated by the canonical pathway, but not by the non-canonical pathway, in the non-hematopoietic cell lines. Many NF-κB binding sites have been identified, most of which are activated by the canonical and non-canonical pathways. The gpκB site may be a unique case of NF-κB responsive elements that may discriminate the canonical pathway from the non-canonical pathway. Among three transcription factor pathways, involving NF-κB, CREB and SRF, which are directly activated by Tax1, Tax1-mediated NF-κB activation has been studied extensively and intensively. This is because ATL cells, including their derivative cell lines, carry constitutively active NF-κB (Yoshida et al., 1982). Studies suggest a close link between

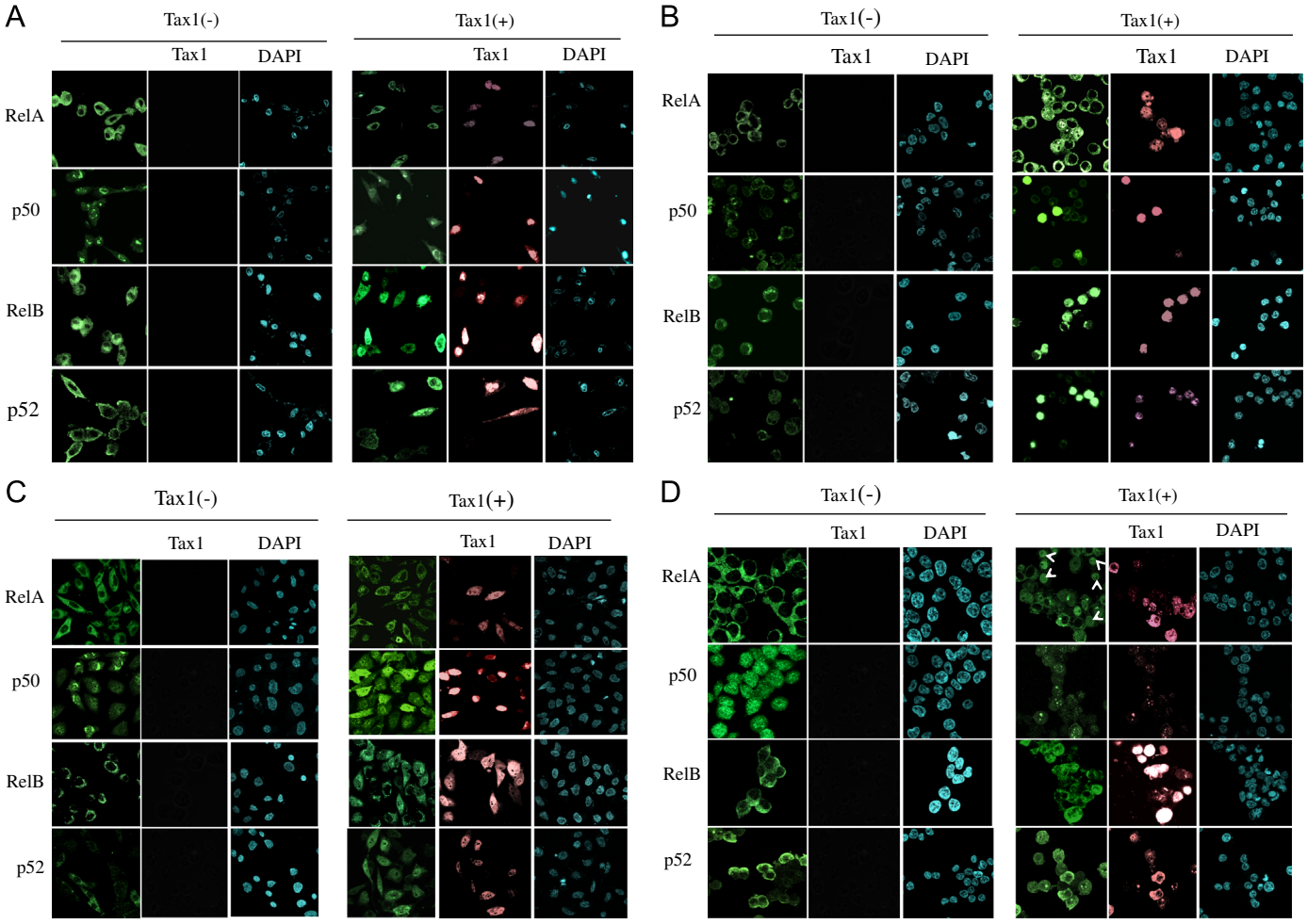

Fig. 5. Expression and function of NF-κB subunits. REF52 (A), Jurkat (B), MG63 (C) and HeLa (D) cells were transfected with or without pMT-2Tax, permealized, stained with the indicated antibodies and observed under a confocal microscope. DAPI was used for nuclear staining. Arrowheads indicate HeLa cells which express RelA in the nucleus without Tax1 expression. For Western blotting, lysates were prepared from REF52 (E) and Jurkat (F) transfected with pMT-2Tax, and MG63 (G) and HeLa (H) cells transfected with pMT-2Tax or cultured with TNFα, electrophoresed and transferred onto membranes. The membranes were treated by ECL system with the indicated antibodies. C23 and β-tubulin were used as marker proteins in nucleic and cytoplasmic fractions, respectively. REF52 cells were cultured with TNFα and examined for IgκB site responses in luciferase reporter assays (I), IκBα phosphorylation in Western blotting (K) and RelA and RelB localization in immunostaining (J). Data are means ± SE. $^{*}P < 0.05$.

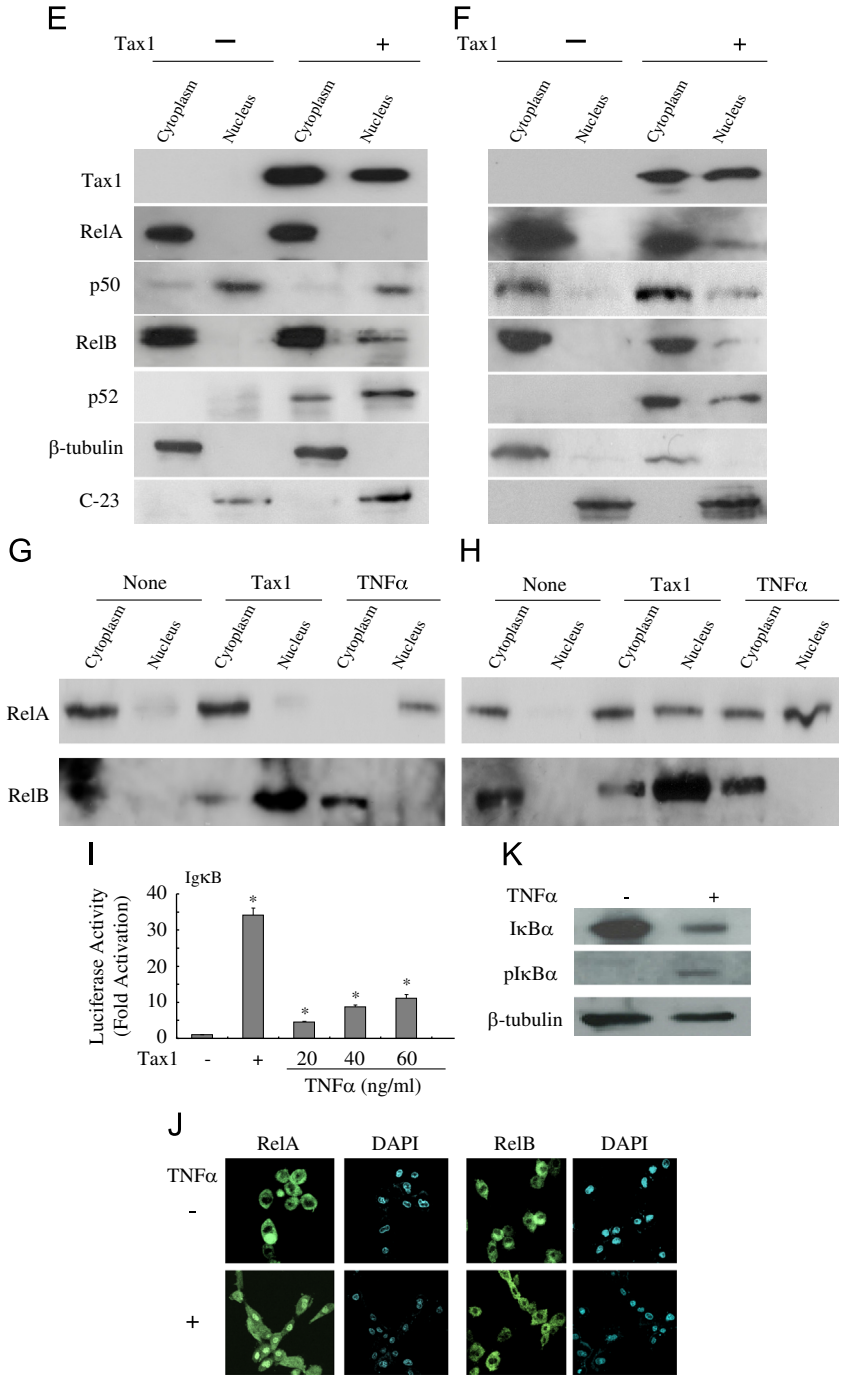

Fig. 5. (*continued*)

Tax1 and malignant transformation (Grossman et al., 1995; Matsuoka and Jeang, 2007; Shoji et al., 2009). In fact, Tax1 mutants lacking the ability to activate NF-κB show poor transforming activity. HTLV-2 Tax2 activates NF-κB similar to Tax1, however malignant transformation by Tax2 has rarely been reported (Higuchi and Fujii, 2009).

In contrast to Tax1, Tax2 immortalizes primary T cells efficiently (our unpublished observations). The efficient immortalization by Tax2 may be attributed to Tax2 specific effects such as activation of nuclear factor of activaed T cells (NF-AT) (Niinuma et al., 2005). We previously demonstrated that Tax1 scarcely induces production of IL-2 in T cells (Mizuguchi et al., 2009). Our results in this study exhibited different activation patterns of the NF-κB binding sites by Tax1 and Tax2; for example, Tax2 activated the Igκ B site less extensively than Tax1 in REF52 cells (Fig. 4). Taking account of the poor ability of Tax2 to activate the non-canonical pathway, this is somewhat unexpected. The difference may be a result that NF-AT is activated by Tax2 but not by either Tax1 or Tax1/2, and also the Igκ B site may be responsive to NF-AT. Similarly, in Jurkat cells, gpκ B response to Tax2 was less than that to Tax1 (Fig. 4). Tax1 activates the canonical and non-canonical pathways in Jurkat cells, inducing full activation of NF-κB. Tax2 and Tax1/2 predominantly activate the canonical pathway, presumably resulting in reduced response of the gpκ B site to Tax2, compared with response to Tax1. The different effects of Tax1 and Tax2 on activation of the κB sites may be closely associated with the differences in pathogenesis by infection with HTLV-1 and HTLV-2.

These assumptions may raise the question why the gpκ B site is not capable of binding of RelB and p52 in non-hematopoietic cell

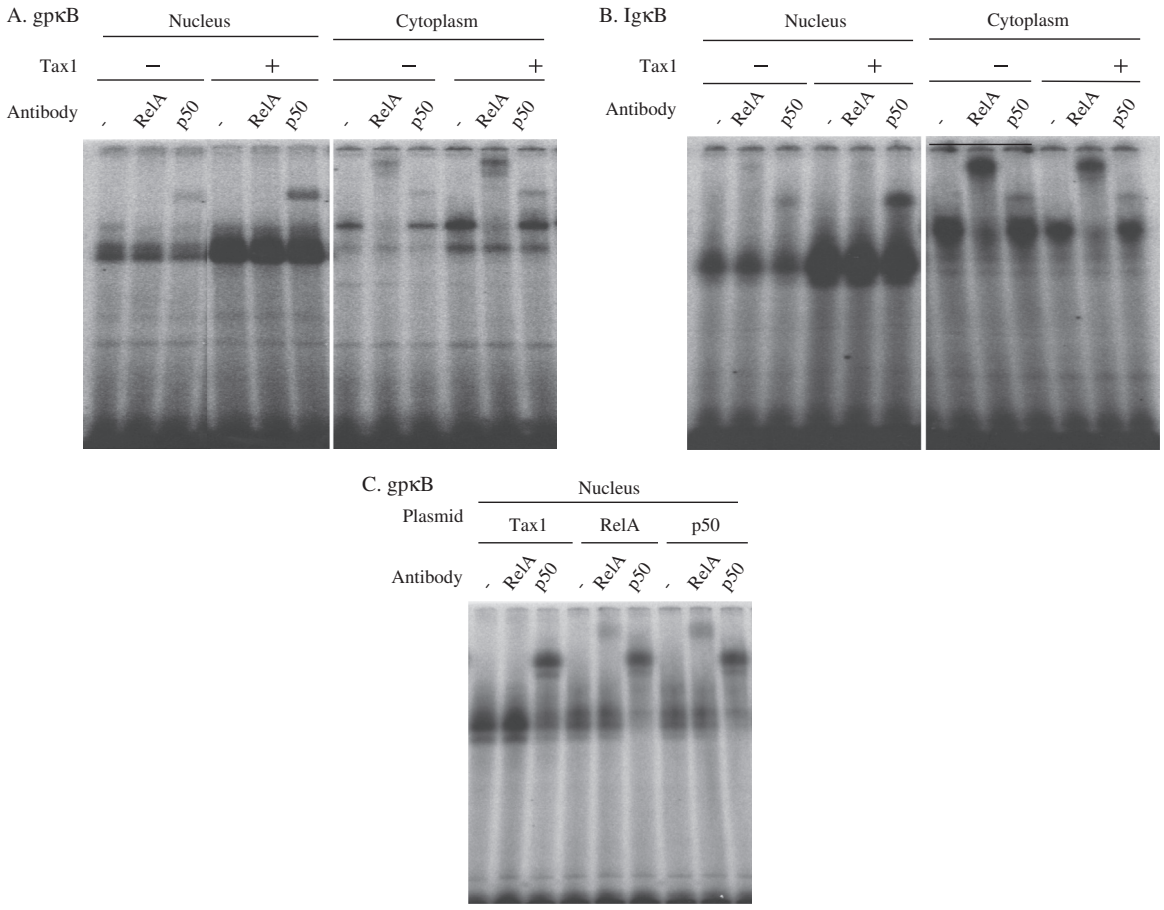

Fig. 6. NF-κB binding to κB sites *in vitro*. The gpκB (A and C) and IgκB (B) sites were used as probes. Probes were labeled and incubated with either nuclear extracts or cytoplasmic extracts prepared from REF52 cells with or without Tax1 expression. Antibodies for RelA and p50 were added to the reaction mixture 1 h prior to the addition of probes. Samples were electrophoresed on polyacrylamide gels and autoradiographed.

lines. It is obvious that Tax1-expressing those cells contained active non-canonical complex RelB/p52, because the IgκB site recruited the RelB/p52 complex (Fig. 7A). However the gpκB site did not show appreciable binding of RelB and p52. At this moment, a solid answer to this question has not been found. Association of RelB and p52 with the gpκB site may be dependent on RelA and p50 binding to the site. This notion may be supported by the result that all four subunits of NF-κB are associated with the gpκB site in Tax1-expressing Jurkat cells (Fig. 7B). Further examination is necessary to clarify this issue.

To gain insight into the mechanism accounting for inactivation of the canonical pathway in non-hematopoietic cell lines, we examined the expression of the canonical pathway subunits RelA and p50. Both molecules were expressed in REF52 cells at a protein level. Although p50 was present in the nucleus and able to bind the gpκB site upon Tax1 expression, RelA was not found in the nucleus even after Tax1 introduction (Figs. 5 and 7). EMSA examination demonstrated complex formation of RelA and p50 with the gpκB site using cytoplasmic lysates, suggesting that the functional complex of RelA and p50 is present in REF52 cells. The gpκB site is capable of RelA/p50 binding. Since Tax1 has been shown to activate the RelA and p50 complex by interaction with IKKγ in the IKK complex (Harhaj et al., 1999; Jin et al., 1999b), we further examined expression of IKKγ by immunostaining with anti-IKKγ antibody. Our observations clearly showed that IKKγ was present in REF52 cells (data not shown). These results imply that an unknown mechanism inhibiting the activation of the canonical pathway complex RelA and p50 may be present in non-hematopoietic cell lines. This mechanism might be related to the process of translocation from the cytoplasm to the nucleus, because RelA was not seen in the nucleus in Tax1 expressing cells.

Cell type-dependent activation of NF-κB by Tax1 is reminiscent of Tax1-mediated activation of the transcription factor E2F. Tax1 activated E2F in hematopoietic cells, however REF52 cells showed little effect of Tax1 on E2F function (Ohtani et al., 2000). Although a cellular factor mediating Tax1-dependent activation of E2F is not known, the Tax1 mutant without the ability to activate NF-κB is also unable to activate E2F (Ohtani et al., 2000). In addition, we observed that Tax2 lacked the ability to activate E2F (our unpublished results). E2F plays critical roles, as a transcription factor, in cell proliferation through progression of the G1 to S phase transition in the cell cycle (Mizuguchi et al., 2011; Ohtani and Nakamura, 2002). Collectively, development of cell transformation by Tax1 may require activation of both the canonical and non-canonical pathways, which are not associated with non-hematopoietic cell lines and Tax2, respectively.

In summary, HTLV-1 Tax1 does not activate the canonical NF-κB pathway in the non-hematopoietic cell lines used in this study and the NF-κB binding site in the OX40L promoter does not respond to the non-canonical pathway in those cells.

Materials and methods

Cell culture

REF52, MG63, HeLa and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal

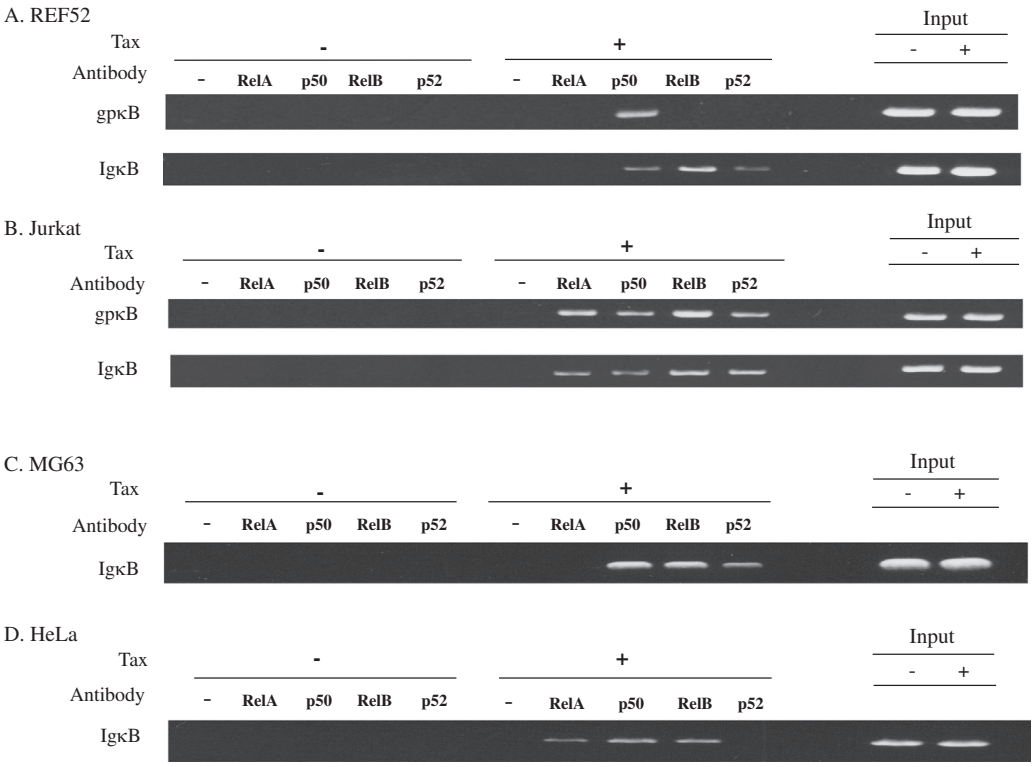

Fig. 7. NF-κB binding to κB sites *in vivo*. Chromatin complexes were prepared from REF52 (A), Jurkat (B), MG63 (C) and HeLa (D) cells transfected with reporter plasmids and pMT-2Tax. After sonication, immunoprecipitation was performed with the antibodies indicated. Precipitated DNA fragments were subjected to PCR with primers specific for the gpκB and IgκB sites in plasmids. The PCR products were 369-bp and 449-bp in length for the gpκB and IgκB sites, respectively.

bovine serum (FBS) with penicillin G (1000 U/ml) and streptomycin (10 μg/ml). Huh7 and HepG2 cells were cultured in DMEM-high glucose (D5796, Sigma-Aldrich) with 10% FBS and antibiotics (Sainz et al., 2009; Wilkening et al., 2003). C-1300 cells were cultured in RPMI1640 medium supplemented with 5% FBS and antibiotics (Fukuhara et al., 1996). Jurkat, K562 and Raji cells were cultured in RPMI1640 with 10% FBS and antibiotics (Karpova et al., 2005; Lozzio and Lozzio,1979; Schneider et al., 1977). TNFα was added to REF52 cell culture for 30 min at a concentration of 40 ng/ml otherwise stated. Cells were cultured at 37 °C in humidified atmosphere with 5% CO_2 in air.

Plasmids

The reporter plasmids used were pIgκB-Luc and pgpκB-Luc carrying three tandem repeats of κB sites in the immunoglobulin light chain (IgκB: 5' –GGGGACTTTCC–3') and gp34 (OX40L) (gpκB: 5'–GGGGAAATTCA–3') genes, respectively, in pGL3-Basic vector (Promega) with the gp34 (OX40L) core promoter (from −31 to +27) (Ohtani et al., 1998). Expression plasmids of NF-κB subunits were R/C CMV RelA for RelA/p65, R/C CMV p50 for p50, R/C CMV RelBM2 for RelB, pCM-p52 for p52, pCn100 for p100 and pEF/c-Rel for c-Rel (Baker et al., 1990; Latimer et al., 1998; Nakayama et al., 1992; Tripathi and Aggarwal, 2006; Yamaoka et al., 1996). The Tax1 expression plasmids pMT-2Tax and pEFneoTax1, and the expression plasmids for wild type Tax2B and a chimeric mutant of Tax1 and Tax2 (Tax1/2) have been described previously (Matsumoto et al., 1997; Shoji et al., 2009). The β-galactosidase expression plasmid pCMV-β-gal was used as an internal control of transfection (Gunning et al., 1987; Matsumoto et al., 1994).

Antibody

Antibodies to NF-κB subunits (RelA, SC-372; p50, SC-114; RelB, SC-19 and p52, SC-848), anti-C23 antibody (H-250), anti-IκBα antibody (SC-371) and anti-β-tubulin antibody (H-235) were purchased from Santa Cruz. Anti-Tax1 mouse monoclonal antibody TAXY-7 is described elsewhere (Tanaka et al., 1991). Anti-phospho-IκBα antibody (5A5) was obtained from Cell Signaling. Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody (eBioscience) and Texas Red-conjugated anti-mouse IgG antibody (Vector) were used for visualization.

Luciferase reporter assay

REF52 and MG63 cells were transfected with 5 μg of plasmid DNA in 2 × HEPES buffered saline with 0.25 M $CaCl_2$ 24 h after cell plating in a 10 cm dish. Jurkat, K562 and Raji cells were transfected with 5 μg of plasmid DNA in DEAE-dextran solution [5 mg/ml in 1 M Tris–HCl (pH 7.4)]. Cells were harvested for reporter assays 48 h post transfection. C-1300, Huh7, HepG2, HeLa and 293T cells were transfected with 1 μg of plasmid DNA using Lipofectamine™ 2000 (Invitrogen) and harvested 24 h after transfection. Luciferase activity was measured with a luminometer (LB 9507, Lumat) using the Luciferase Assay System (Promega) and the activity of luciferase was normalized to β-galactosidase activity which was measured by absorption at 410 nm, or to protein content which was determined by absorption at 750 nm with a spectrophotometer (DU 64, Beckman). Assays were performed at least three times in triplicate. The means ± SE are presented.

Small-interfering RNA (siRNA)

REF52 cells was plated on a 12 well plate and incubated with siRNA (Santa Cruz) for RelB (SC-36403), p52 (SC-36043), RelA (SC-61876) and p50 (SC-156175) at a final concentration of 10 nM in Lipofectamine™ RNAiMAX (Invitrogen) at 37 °C for 6 h. Cells were cultured in medium with 10% FBS and antibiotics for 24 h, transfected with pIgκB-Luc along with pMT-2Tax in Lipofectamine 2000. Cells were further cultured for 24 h and harvested for luciferase activity measurement.

Electrophoretic mobility shift assay (EMSA)

Nuclear and cytoplasmic lysates were prepared from cells transfected with or without the indicated expression plasmids as described (Adachi et al., 1998). From each lysate, 5 μg protein were incubated with [^{32}P]-labeled probes containing κB binding (IgκB and gpκB) sites in DNA-binding buffer [13 mM HEPES (pH 7.8), 8% glycerol, 65 mM NaCl, 1 mM DTT, 0.15 mM EDTA and 1 μg of poly (dI-dC)]. In supershift assays, antibodies (1 μg) were added 1 h before probe addition. The reaction products were separated on 4% polyacrylamide gels in 5 × TBE buffer [450 mM Tris (pH 8.0), 450 mM boric acid and 10 mM EDTA (pH 8.0)] for 4 h at 200 V. Gels were dried and autoradiographed.

Chromatin immunoprecipitation (ChIP) assay

Cells were fixed in 10% formaldehyde solution for 15 min at 37 °C. Crosslinked cells were harvested, lysed in a buffer [50 mM Tris–HCl (pH 8.1), 1% SDS and 5 mM EDTA] containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated 6 times of 30 s at output 4 of Duty 80 (UD-201, Tomy). The supernatant liquid was collected by centrifugation and incubated with dilution buffer [20 mM Tris–HCl (pH 8.1), 2 mM EDTA, 150 mM NaCl and 1% Triton X-100] containing PMSF with 2 μg of salmon sperm DNA, 20 μl of normal rabbit serum (Dako) and 45 μl of rProtein A Sepharose (GE Health Bio-Science) for 2 h at 4 °C. After rProtein A Sepharose was removed from samples, 2 μg of antibody (anti-RelA, anti-p50, anti-RelB or anti-p52 antibody) was added to each sample and incubated on a rotating platform for 24 h at 4 °C. Salmon sperm DNA (2 μg) and 45 μg of rProtein A Sepharose were added to each sample and incubated for 1 h at 4 °C. DNA fragments were eluted, purified and subjected to PCR with specific primers (IgκB site, forward primer, 5'–TGGAGCGGCCGCAATAAAATA–3', reverse primer, 5'-GGGCGGAGAATGGGCGGAAACT-3' gpκB site, forward primer, 5'-CGGGCCTCTTCGCTATTAG-3' reverse primer, 5'-GCGCCGGGCCTTTCTTTATGTTTT-3'). PCR products were electrophoresed on 2% agarose gels.

Western blotting assay

Cells transfected with or without pMT-2Tax were harvested for preparation of nuclear and cytoplasmic lysates. Cells were lysed with buffer A [10 mM HEPES (pH 7.8), 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% NP-40, 1 mM DTT and 0.5 mM PMSF] and centrifuged to separate nuclei from supernatants. The resultant supernatants were used as sources of cytoplasmic lysates after addition of glycerol and KCl at final concentrations of 20% and 100 mM, respectively. Nuclear lysates were prepared by extraction from nuclei with buffer C [50 mM HEPES (pH 7.8), 420 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 2% glycerol, 1 mM DTT and 0.5 mM PMSF]. Electrophoresis was performed with 20 μg of protein on 9% polyacrylamide gels. Proteins were blotted into membranes (AE-6667, Atto). Membranes were immunostained and visualized with antibodies using Enhanced Chemi Luminescence (ECL) (Amersham Biosciences).

Immunofluorescence staining

Cells were plated on cover glasses, fixed with 10% paraformaldehyde in PBS, and permeabilized with 0.4% Triton X-100 in PBS. Cells were incubated in 3% bovine serum albumin fraction V in PBS for 30 min and further incubated with mouse anti-Tax1 monoclonal antibody (TAXY-7) at 5 μg/ml for 1 h followed by the addition of Texas Red-conjugated anti-mouse IgG antibody. Anti-RelA, anti-p50, anti-RelB and anti-p52 antibodies were used at 5 μg/ml with FITC-conjugated anti-rabbit IgG antibody. Cells were examined with a laser scanning confocal microscope (FV10i, Olympus).

Statistical analysis

Differences in means between samples and controls were assessed for statistical significance by the student's t-test. Values less than 0.05 are taken statistically significant.

Acknowledgments

We thank J. Inoue and S. Yamaoka for providing NF-κB expression plasmids, and W. Hall for a Tax2 clone. We are grateful to L. Preston for critical reading of the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.04.032.

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